

Osteoarthritis and Cartilage



Interleukin 6 mediates selected effects of Notch in chondrocytes



S. Zanotti^{†‡*}, E. Canalis^{†‡}

[†] Department of Research, Saint Francis Hospital and Medical Center, Hartford, CT 06105, USA

[‡] The University of Connecticut School of Medicine, Farmington, CT 06030, USA

ARTICLE INFO

Article history:

Received 13 March 2013

Accepted 3 August 2013

Keywords:

Notch

Mmp13

Il6

Col10a1

Acan

Chondrocytes

SUMMARY

Objective: Notch receptors determine cell fate by regulating transcription, an event mediated by the Notch intracellular domain (NICD), which is generated by proteolysis brought about by Notch–ligand interactions. Since Notch activation or exposure to interleukin (Il)6 have similar effects in chondrocytes, we explored whether interleukin 6 (Il6) contributes to the mechanisms of Notch action in these cells.

Method: NICD was overexpressed in primary chondrocytes from *Rosa^{Notch}* mice, where the *Rosa26* promoter precedes a *loxP*-flanked STOP cassette followed by the NICD coding sequence. Cells were infected with adenoviral vectors expressing Cre to induce NICD or green fluorescent protein (GFP) as control. Gene expression was determined by quantitative reverse transcription-polymerase chain reaction (qRT-PCR). Il6 protein concentration in the culture media was determined by enzyme-linked immunosorbent assay (ELISA). To test the mechanisms of Notch action on *Il6* expression, cells were transfected with a fragment of the *Il6* promoter or control vector pGL3, or transcriptionally arrested with 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole. Il6 was inhibited with a neutralizing antibody, whereas a normal immunoglobulin G (IgG) was used as control.

Results: NICD induced *Il6* mRNA and protein, and transactivated the *Il6* promoter without affecting *Il6* mRNA stability. Il6 neutralization had no impact on gene expression under basal conditions, and did not modify the effects of NICD on sex determining region-Y-related high mobility group-box gene (*Sox*)9, collagen type II α1 (*Col2a1*) and collagen type X α1 (*Col10a1*) expression. Conversely, Il6 neutralization opposed aggrecan (*Acan*) suppression and prevented matrix metalloprotease (*Mmp*)13 induction by NICD.

Conclusion: Il6 mediates suppression of *Acan* and induction of *Mmp13* expression by Notch in chondrocytes.

© 2013 Osteoarthritis Research Society International. Published by Elsevier Ltd. All rights reserved.

Introduction

The Notch1 to Notch4 receptors, and the jagged (Jag)1 and 2, and delta-like (Dll)1, 3 and 4 ligands are transmembrane proteins that relay signals between adjacent cells, thereby determining cell fate and function¹. Interactions of Notch with Jag or Dll result in sequential proteolytic cleavages by extracellular metalloproteases and by the γ-secretase protein complex, leading to the release of the intracellular domain of Notch (NICD) to the cytoplasm². NICD translocates to the nucleus and associates with the DNA binding protein Epstein–Barr virus latency C-promoter binding factor 1, suppressor of hairless, and lag1 (CSL), also known as Rbpjk in mice,

and with mastermind-like proteins. Subsequently, the transcriptional repressors associated with Rbpjk are displaced by transcriptional activators, resulting in the induction of Notch target genes, such as those encoding for hairy enhancer of split (Hes) and Hes related with YRPW-motif (Hey)^{3,4}.

Chondrocytes are cartilage forming cells of mesenchymal origin, and proliferation and differentiation of growth plate chondrocytes results in linear growth of long bones⁵. Chondrocytes in articular cartilage derive from mesenchymal precursors found at the articular surface, and under physiological conditions, these cells express proteoglycan (*Pr*)4, sex determining region-Y-related high mobility group-box gene (*Sox*)9, aggrecan (*Acan*) and collagen type II α1 (*Col2a1*) and preserve the function of the extracellular matrix^{6,7}. Changes in the synovial fluid concentration of *Pr*4, and suppressed *Sox*9, *Acan* and *Col2a1* transcript levels associated to increased expression of collagen type X α1 (*Col10a1*) by articular chondrocytes, are observed when cartilage integrity is compromised^{8–11}. These events are coupled to the induction of gene

* Address correspondence and reprint requests to: S. Zanotti, Department of Research, Saint Francis Hospital and Medical Center, 114 Woodland Street, Hartford, CT 06105-1299, USA. Tel: 860 714-5250; Fax: 860 714-8053.

E-mail address: szanott@stfranciscare.org (S. Zanotti).

markers of matrix degradation, such as a disintegrin-like and metalloproteinase with thrombospondin type 1 motif (*Adamts*)4 and matrix metalloproteinase (*Mmp*)13, and to the expression of mediators of inflammation, such as interleukin (Il)6^{12–14}.

Although Notch signaling suppresses chondrogenesis and differentiation of hypertrophic chondrocytes, under selected conditions, overexpression of NICD in chondrocytes induces *Col10a1* and *Mmp13* transcripts^{15–22}. Increased expression of components of the Notch signaling pathway and nuclear localization of the intracellular domains of NOTCH1 and NOTCH2 are observed in human osteoarthritic chondrocytes, indicating that activation of Notch signaling is associated with cartilage degeneration^{23–25}. Accordingly, inactivation of *Rbpjk* in chondrocytes or suppression of Notch by a γ -secretase inhibitor prevents surgically induced cartilage degeneration, suggesting that activation of Notch signaling is detrimental to the integrity of articular cartilage²⁵. However, the mechanisms that mediate the effects of Notch on chondrocyte function are understood partially.

Notch induces interleukin 6 (Il6) expression in cells of mesenchymal origin, and exposure of bovine, rabbit, and human chondrocytes to Il6 phenocopies selected effects of Notch in murine chondrocytes^{26–29}. In this study, we investigated whether Notch regulates Il6 expression in chondrocytes and whether Il6 mediates the effects of Notch in these cells. To this end, primary chondrocyte-enriched cells were harvested from *Rosa^{Notch}* mice where Cre recombination induces the expression of NICD following excision of a loxP-flanked STOP cassette, placed between the *Rosa26* promoter and the NICD coding sequence³⁰.

Methods

Primary chondrocyte-enriched cell cultures

Rosa^{Notch} mice, generated by D.A. Melton (Harvard University, Cambridge, MA), were obtained from Jackson Laboratories (Bar Harbor, ME)³⁰. Chondrocyte-enriched cells were obtained from 3 to 4 day old male and female littermate *Rosa^{Notch}* or wild-type C57BL/6 mice and cultured independently in order to retain the individual identity of the donor. The distal epiphysis of the femur, tibia, humerus, ulna and radius, and the proximal epiphysis of the tibia and ulna were dissected under a Unitron Z850 stereo microscope (Unitron, Commack, NY), and trabecular bone removed to limit contamination from osteoblastic cells. Cartilage was transferred to high glucose Dulbecco's modified Eagle's medium (DMEM, Life Technologies, Grand Island, New York) and digested with 0.25% trypsin in 0.9 mM EDTA (Life Technologies) for 40 min at 37°C with continuous mixing. Subsequently, cartilage was exposed to DMEM containing 10% fetal bovine serum (FBS, Atlanta Biologicals, Norcross, GA) and 200 U/ml of type II collagenase from *Clostridium histolyticum* (Worthington Biochemical Corporation, Lakewood, NJ) for 2–4 h at 37°C with continuous mixing. Following digestion, tissue debris was removed by straining through a 70 μ m membrane and chondrocyte-enriched cells were collected by centrifugation at 500 g for 5 min. Cells were seeded at a density of 50,000 cells/cm² and cultured in DMEM supplemented with 10% FBS at 37°C in a humidified 5% CO₂ incubator^{21,31}. Experimental protocols were approved by the Animal Care and Use Committee of Saint Francis Hospital and Medical Center.

Adenoviral infection

Primary chondrocyte-enriched cells from *Rosa^{Notch}* mice were transferred to DMEM containing 2% FBS for 1 h and exposed overnight to 100 multiplicity of infection of replication defective recombinant adenoviruses (Ads). An adenoviral vector expressing

Cre recombinase under the control of the cytomegalovirus (CMV) promoter (Ad–CMV–Cre, Vector Biolabs, Philadelphia, PA) was used to excise the STOP cassette and allow NICD expression. An adenoviral vector where the CMV promoter directs expression of green fluorescent protein (GFP; Ad–CMV–GFP, Vector Biolabs) was used as control. Following infection, primary chondrocyte-enriched cells were allowed to recover for 24–48 h and cultured in the presence of DMEM containing 10% FBS²¹. At confluence, cultures were exposed to 100 μ g/ml ascorbic acid (Sigma–Aldrich, Saint Louis, MO) to prevent loss of chondrocyte phenotype and promote acquisition of a mature chondrocyte phenotype³². Activation of Notch signaling in *Rosa^{Notch}* cells expressing Cre recombinase and the effects of Notch on the chondrocyte phenotype were confirmed in independent cultures from male or female mice and the effects of Notch were not sexually dimorphic (data not shown)^{21,33–36}. Therefore, observations from cell cultures obtained from mice of either sex were pooled for analysis of results.

Cytochemical staining, enzyme-linked immunosorbent assay (ELISA) and Il6 neutralization

To determine the formation of chondrogenic and mineralized nodules, cultures from *Rosa^{Notch}* mice were fixed for 10 min at room temperature in 3.7% formaldehyde in phosphate buffered saline and subsequently stained with 1% alcian blue in 3% acetic acid, or 2% alizarin red in H₂O (all from Sigma–Aldrich). Images were acquired with a Coolpix 995 digital camera mounted on an Eclipse TS100 inverted microscope equipped with contrast phase lenses (all from Nikon Inc., Melville, NY).

To explore whether NICD induces Il6 protein levels, confluent *Rosa^{Notch}* chondrocyte-enriched cells were cultured for 3 days before exposure for 24 h to DMEM containing 100 μ g/ml ascorbic acid. Il6 concentration in the medium was measured with a mouse Il6 ELISA kit, in accordance to the manufacturer's instructions (BD Biosciences, San Jose, California).

In selected experiments, *Rosa^{Notch}* chondrocyte-enriched cells were exposed to DMEM for 6 h and subsequently exposed DMEM containing 1 μ g/ml neutralizing murine monoclonal immunoglobulin G (IgG) against Il6 (clone B-E8; EMD Millipore, Billerica, MA) or control murine normal IgG (Santa Cruz Biotechnology, Santa Cruz, CA).

Transient transfections

To assess whether NICD regulates Il6 transcription in chondrocytes, chondrocyte-enriched cells from *Rosa^{Notch}* mice were transiently transfected with a 1.3 kilobase (kb) fragment of the murine Il6 promoter cloned into pGL3 basic, upstream of the luciferase (Luc) coding sequence (Il6–Luc; D.L. Allen, University of Colorado Boulder, Boulder, CO)³⁷. To induce Notch signaling in wild-type chondrocyte-enriched cells, a 2.4 kb DNA fragment containing the murine NICD coding sequence (J.S. Nye, Columbia University, New York City, NY) was cloned in the pcDNA3.1 expression vector (Life Technologies, Carlsbad, CA) downstream of the CMV promoter to generate pcDNA–NICD³⁸. To determine the effects of NICD on Notch transactivation in wild-type chondrocytes, cells were transiently transfected with pcDNA–NICD or with pcDNA3.1, as control, and co-transfected with a construct where six multimerized dimeric CSL binding sites linked to the β -globin basal promoter govern luciferase expression (12 \times CSL–Luc; L.J. Strobl, Munich, Germany)³⁹. To confirm the effects of NICD on the transactivation of the Il6 promoter, wild-type chondrocytes transiently transfected with pcDNA–NICD or with pcDNA3.1 were co-transfected with Il6–Luc. To correct for transfection efficiency, cells were co-transfected with a construct where the CMV promoter directs β -galactosidase

expression (Clontech, Mountain View, CA). *Rosa^{Notch}* chondrocytes were exposed to a 3 μ l/2 μ g mix of FuGene6 (Roche, Indianapolis, IN) and DNA for 16 h before adenoviral infection with Ad–CMV–Cre or Ad–CMV–GFP. Wild-type chondrocyte cultures at 70% confluence were exposed to a 3 μ l/2 μ g mix of X-tremeGENE 9 (Roche) and DNA for 16 h. Cells were harvested after 48 h in luciferase extraction buffer (Roche), and luciferase and β -galactosidase activities were measured on an Optocomp luminometer (MGM Instruments, Hamden, CT), according to manufacturer's instructions (Roche).

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Total RNA from chondrocyte-enriched cells from *Rosa^{Notch}* mice was extracted with the RNeasy mini kit, according to manufacturer's instructions (Qiagen, Valencia, CA) and changes in gene expression determined by qRT-PCR. Half to one microgram of total RNA were reverse-transcribed using the iScript cDNA synthesis kit (BioRad, Hercules, CA), according to manufacturer's instructions, and amplified in the presence of specific primers (Table 1; all from Integrated DNA Technologies (IDT)), and iQ SYBR Green Supermix (BioRad) at 60°C for 35 cycles. mRNA copy number was estimated by comparison to a 10-fold serial dilution of cDNA for *Acan*, *Adamts4*, *Col10a1*, *Il6*, *Mmp13* and *Sox9* (all from Thermo Scientific, Rockford, IL), *Col2a1*, *Hes1*, ribosomal protein 1 (*Rpl38*) (all from American Type Culture Collection, Manassas, VA), *Prg4* (Source BioScience, Nottingham, United Kingdom), *Hey1* and *Hey2* (both from T. Iso, University of Southern California, Los Angeles, CA) and *HeyL* (*HeyL*; from D. Srivastava, University of Texas Southwestern Medical Center, Dallas, TX)^{4,40–42}. Reactions were

conducted in a CFX96 real time PCR detection system (BioRad), fluorescence monitored during every PCR cycle at the annealing step and specificity of the reaction assessed by analysis of melting curves.

RNA decay experiments

To establish the effects of NICD on the stability of *Il6* mRNA, RNA polymerase 2 was inhibited in confluent chondrocyte-enriched cell cultures from *Rosa^{Notch}* mice by exposure to 300 μ M 5,6-dichloro-1- β -D-ribofuranosylbenzimidazole (DRB, BioMol, Plymouth Meeting, PA). *Il6* mRNA levels at various times following the exposure to DRB were determined in total RNA extracts by qRT-PCR analysis. The slopes of mRNA decay were established by fitting the log 10 of the percentage of *Il6* transcript levels corrected for *Rpl38* expression over corrected *Il6* transcripts before exposure to DRB against time by linear regression.

Statistical analysis

Estimates and associated uncertainty are expressed as means and 95% confidence interval, respectively. Each observation represents an independent culture from individual mice and is the average of two technical replicates. Normality of data distribution was determined with 95% confidence by the Shapiro–Wilk test. Significance of statistical differences was tested for pairwise comparisons by the Student's *t*-test with a *P* < 0.05 and for multiple comparisons by applying the Bonferroni correction with a *P* < 0.0250⁴³. Statistical differences between slopes of regression lines of corrected *Il6* mRNA levels against time were determined by analysis of covariance⁴⁴. Statistical analyses were performed using Microsoft Office Excel 2003 SP2 (Microsoft, Redmond, WA) or PASW software version 18.0.0 (IBM, Armonk, NY).

Results

NICD induces *Il6* by a transcriptional mechanism in chondrocyte-enriched cells

Exposure to *Il6* recapitulates selected effects of Notch in chondrocytes, and we tested whether Notch signaling regulates *Il6* expression in chondrocyte-enriched cells from *Rosa^{Notch}* mice infected with Ad–CMV–Cre to induce NICD or with control Ad–CMV–GFP^{26–29}. Analysis of mRNA levels by qRT-PCR revealed that activation of Notch signaling induced a seven fold increase in *Il6* expression [Fig. 1(A)]. To establish whether induction of *Il6* transcripts by Notch translates into increased *Il6* production, levels of *Il6* protein were measured by ELISA in the supernatant of *Rosa^{Notch}* chondrocytes. *Il6* concentration in the culture medium of controls and of cells overexpressing NICD was about 20 pg/ml and 290 pg/ml, respectively [Fig. 1(B)], demonstrating that Notch caused a 15-fold increase of *Il6* protein levels and confirming that activation of Notch signaling in chondrocytes induces *Il6*.

To study the mechanisms that mediate induction of *Il6* by Notch, the effects of Notch activation on the transactivation of a transiently transfected 1.3 kb fragment of the *Il6* promoter (*Il6*–Luc) or on control pGL3 vector were tested in chondrocyte-enriched cells from *Rosa^{Notch}* littermate mice. Activity of pGL3 was not induced by Notch in comparison to controls, indicating that pGL3 does not contain regulatory DNA elements responsive to Notch signaling in chondrocytes. In control cells, activity of *Il6*–Luc was about 100-fold greater than the activity of pGL3, suggesting that under control conditions the *Il6* promoter is transactivated in chondrocytes [Fig. 1(C)]. In accordance with the stimulatory effects of Notch on *Il6* mRNA levels, *Il6*–Luc was transactivated in the context of Notch

Table 1

Primers used for qRT-PCR. Forward (Fwd) and reverse (Rev) primers used to determine changes in gene expression by qRT-PCR. GenBank accession numbers for the transcript variants recognized by primer pairs are indicated.

Gene	Strand	Primer sequence	GenBank accession number
<i>Acan</i>	Fwd	5'-ATGGTCTTCTATGACATACACTCC CCG-3'	NM_007424
	Rev	5'-TTGTACAGCCGCCACCAAGG-3'	
<i>Adamts4</i>	Fwd	5'-GATGTGTGCAAGCTTACCT-3'	NM_172845
	Rev	5'-CATCCGTAACCTTTGGAGA-3'	
<i>Col2a1</i>	Fwd	5'-GACCCAAACACTTTTCAACCGCAGT-3'	NM_031163;
	Rev	5'-TCATCAGGTGTCAGGTGAGCATT-3'	
<i>Col10a1</i>	Fwd	5'-CAGGCTTTCTGGGATGCCGCTTGT-3'	NM_009925
	Rev	5'-GGGCACCTACTGCTGGGTAA-3'	
<i>Hes1</i>	Fwd	5'-ACCAAAGACGGCTCTGAGCACAGAA AGT-3'	NM_008235
	Rev	5'-ATTCTTGCCTTTCGCCTCTT-3'	
<i>Hey1</i>	Fwd	5'-ATCTCAACAACATCGCATCCAGC-3'	NM_010423
	Rev	5'-GTGTGGGTGATGTCCGAAGG-3'	
<i>Hey2</i>	Fwd	5'-AGCGAGAACAAATTACCCTGGGCAC-3'	NM_013904
	Rev	5'-GAGGTAGTTGTCGGTGAATTGG-3'	
<i>HeyL</i>	Fwd	5'-CAGTAGCCTTTCTGAATTGGAC-3'	NM_013905
	Rev	5'-GCTTGGAGGAGCCCTGTTTCT-3'	
<i>Il6</i>	Fwd	5'-CGGCCTTCCCTACTTCAAGTCCG-3'	NM_031168
	Rev	5'-CAGGTCTGTTGGAGTGGTATCC-3'	
<i>Mmp13</i>	Fwd	5'-GGAAGACCTTGTGTTGCAGAGC-3'	NM_008607
	Rev	5'-CACTGTAGACTTCTCAGGATTCCTG-3'	
<i>Prg4</i>	Fwd	5'-CGCCTTTTCCAAAGATCAATACTA-3'	NM_021400;
	Rev	5'-GTGGTAATGTCTCTGTCTGT-3'	
<i>Rpl38</i>	Fwd	5'-AGAACAAGGATAATGTGAAGTTCAAG GTTC-3'	NM_001048057;
	Rev	5'-CTGCTTCAGCTTCTCTGCCTTT-3'	
<i>Sox9</i>	Fwd	5'-CCTACTACAGTCAGCAGCCG-3'	NM_011448
	Rev	5'-GGGTTCATGTAAGTGAAGTTGGA-3'	

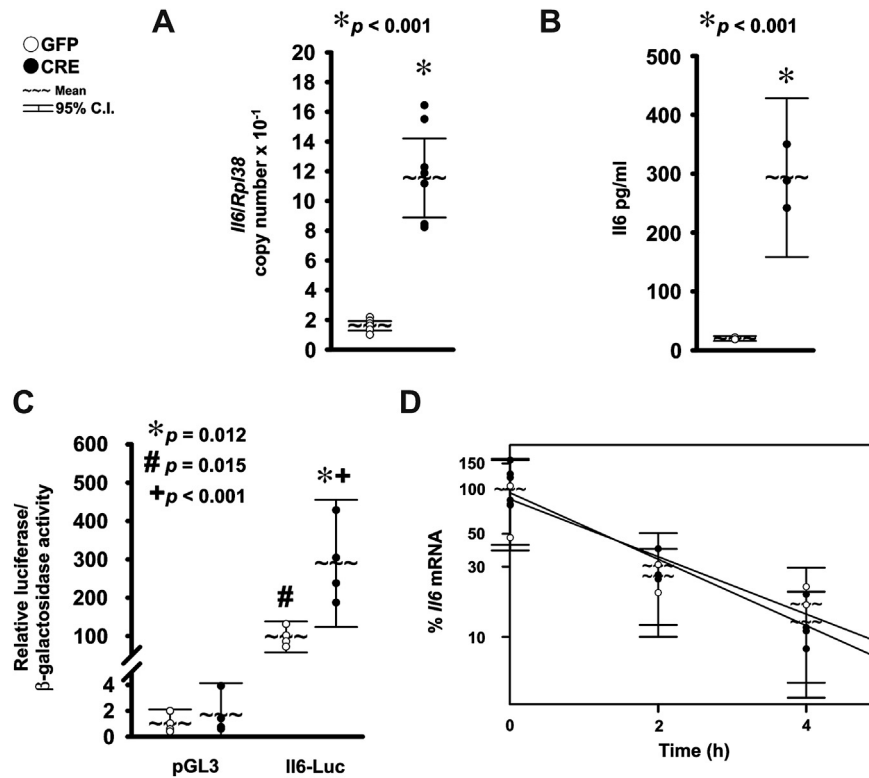


Fig. 1. NICD induces IL6 expression by transcriptional mechanisms. Primary *Rosa^{Notch}* chondrocyte-enriched cells were infected with Ad–CMV–Cre (CRE, full circles) to induce NICD or with control Ad–CMV–GFP (GFP, empty circles). In panel A, total RNA was extracted 3 days after adenoviral infection, and changes in gene expression analyzed by qRT-PCR. Data are expressed as ratio of *Il6* copy number over *Rp138* copy number. Observations represent cultures from individual mice, $n = 8$. * indicates differences between CRE and GFP. In panel B, IL6 concentration in the culture medium was determined by ELISA. Observations represent cultures from individual mice, $n = 3$. * indicates a difference between CRE and GFP. In panel C, cells were transfected with the pGL3 luciferase expression vector or with a fragment of the *Il6* promoter cloned in pGL3 (IL6–Luc), and a β-galactosidase expression vector 24 h before adenoviral infection and harvested after 48 h. Data are expressed as luciferase activity corrected for β-galactosidase activity, relative to corrected luciferase activity in control cells transfected with pGL3. Observations represent cultures from individual mice $n = 4$. * indicates differences between CRE and GFP. # indicates a difference between IL6–Luc and pGL3 activity in cells expressing GFP. + indicates a difference between IL6–Luc and pGL3 activity in cells expressing CRE. In panel D, chondrocytes were transcriptionally arrested with DRB (time 0), and total RNA harvested at the indicated times was amplified by qRT-PCR. Data are percentage of *Il6* mRNA copy number corrected for *Rp138* copy number, relative to corrected expression of *Il6* at the time of exposure to DRB, plotted vs time (h). Intercept and slope of regression lines were calculated by linear regression of corrected *Il6* expression over time.

induction. The extent of this effect was less pronounced than the induction of IL6 mRNA and protein levels by Notch, indicating that additional regulatory regions present in the intact *Il6* locus determine the magnitude of *Il6* induction by Notch. To assess whether Notch regulates the stability of *Il6* transcripts, *Il6* expression was determined by qRT-PCR in *Rosa^{Notch}* chondrocyte-enriched cells exposed to DRB, an inhibitor of mRNA transcription. The estimated half-life of the *Il6* transcript was 1.2 h, and Notch did not affect the rate of *Il6* mRNA decay [Fig. 1(D)], indicating that post-transcriptional mechanisms do not mediate the effects of Notch on *Il6* expression.

To induce Notch activity in chondrocytes by alternate mechanisms, chondrocyte-enriched cells from wild-type C57BL/6 mice were transiently transfected with pcDNA–NICD or with control pcDNA3.1. To determine whether pcDNA–NICD activates the Notch signaling pathway, cells were co-transfected with pGL3 or with the 12 \times CSL–Luc reporter. Transient NICD overexpression had no impact on pGL3 activity whereas it induced 12 \times CSL–Luc transactivation, confirming activation of Notch signaling (Fig. 2). Under basal conditions, IL6–Luc activity was about eight fold higher than pGL3 activity, whereas in the context of Notch induction IL6–Luc transactivation was about four fold higher than in the presence of pcDNA. These results suggest that the *Il6* promoter has basal activity in chondrocytes and confirm that Notch induces *Il6* expression by transcriptional mechanisms.

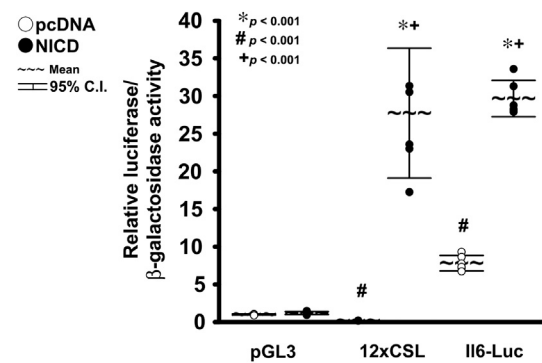


Fig. 2. Transient overexpression of NICD transactivates the *Il6* promoter. Primary chondrocyte-enriched cells from littermate wild-type C57BL/6 mice were transfected with pcDNA–NICD (NICD, full circles) to induce NICD or with control pcDNA3.1 (pcDNA, empty circles). Cells were co-transfected with the pGL3 luciferase expression vector, the 12–CSL–Luc reporter (12 \times CSL) or a fragment of the *Il6* promoter cloned in pGL3 (IL6–Luc), and a β-galactosidase expression vector. Chondrocytes were harvested after 48 h. Data are expressed as luciferase activity corrected for β-galactosidase activity, relative to corrected luciferase activity in control cells transfected with pGL3. Observations represent cultures from individual mice $n = 6$. * indicates differences between NICD and pcDNA. # indicates a difference between 12 \times CSL, or IL6–Luc, and pGL3 activity in cells transfected with pcDNA. + indicates a difference between 12 \times CSL, or IL6–Luc, and pGL3 activity in cells transfected with NICD.

Il6 neutralization opposes the effects of NICD on *Mmp13* and *Acan* expression

Exposure of chondrocytes to Il6 phenocopies selected effects of Notch signaling, and we asked whether Il6 mediates the effects of Notch on the expression of chondrocyte gene markers^{27–29}. To investigate this possibility, chondrocyte-enriched cells from *Rosa^{Notch}* mice were exposed to a neutralizing murine monoclonal IgG raised against Il6 or murine IgG, either in the context of Notch activation or under basal conditions.

Notch induced *Hey1*, *Hey2* and *HeyL*, and to a lesser extent, *Hes1* transcripts, either in cells exposed to the neutralizing Il6 antibody or to normal IgG (Fig. 3), demonstrating that inhibition of Il6 does not alter Notch transactivation in chondrocyte-enriched cells. In agreement with previous results, Notch activation suppressed *Sox9* and *Col2a1* mRNA levels and induced *Col10a1* expression in cells exposed to normal IgG^{20,21,45}. Similar results were obtained in the presence of the Il6 neutralizing antibody, indicating that Il6 is not required for these effects of Notch in chondrocytes. Levels of *Il6* mRNA were not affected by the neutralizing Il6 antibody, either under basal conditions or in the context of NICD overexpression, excluding the possibility that the neutralizing Il6 antibody precludes induction of *Il6* expression by Notch (Fig. 4). Inhibition of Il6 in the absence of Notch had no impact on *Sox9*, *Col2a1* and *Col10a1* expression, suggesting that basal Il6 levels are not required for expression of these chondrocyte gene markers.

Although Notch activation had no effect on *Prg4* transcripts, Notch suppressed *Acan* mRNA levels and this effect was opposed partially by Il6 neutralization, indicating that Notch inhibits expression of selected gene markers of chondrocyte function and this effect is mediated in part by Il6. Notch activation did not affect *Adamts4* expression, whereas it increased *Mmp13* transcripts levels, confirming that under selected conditions NICD overexpression

induces gene markers of cartilage matrix degradation (Fig. 5)^{20,21}. Neutralization of Il6 opposed the stimulatory effects of Notch on *Mmp13* expression, indicating that Il6 is required for this effect of Notch in chondrocytes (Fig. 5). Il6 neutralization in cells expressing GFP did not affect *Prg4*, *Acan*, *Adamts4* and *Mmp13* transcript levels, confirming that Il6 is dispensable for expression of selected chondrocyte gene markers.

Discussion

In this study, we explored the mechanisms that mediate the regulation of gene expression by the Notch signaling pathway in chondrocytes. Several studies have established Notch as a suppressor of chondrogenesis *in vivo*, and this effect is consistent with the role of Notch as an inhibitor of mesenchymal cell differentiation¹. However, the role of Notch signaling in differentiated cells of the chondrocyte lineage is controversial, since both suppression of hypertrophic differentiation and elongation of the hypertrophic zone of the growth plate have been described as effects of NICD overexpression under the control of the *Col2a1* promoter^{18,20,22}. The validity of the *Rosa^{Notch}* system to study the effects of Notch signaling in primary chondrocyte-enriched cell cultures was confirmed by the induction of Notch target genes following infection with Ad–CMV–Cre (data not shown). In agreement with previous findings, Notch induced expression of *Hey1*, *2* and *L* to a greater extent than *Hes1* (data not shown)²¹.

We confirmed that induction of Notch signaling in primary chondrocytes, irrespective of donor sex, inhibits progression to a mature chondrocyte phenotype while inducing expression of *Col10a1* and *Mmp13*^{20,21}. In previous work we reported that chondrocyte-enriched cell cultures present minimal expression of osteoblast gene markers, suggesting the presence of osteoblastic cells²¹. Under selected conditions, osteoblasts express *Mmp13*,

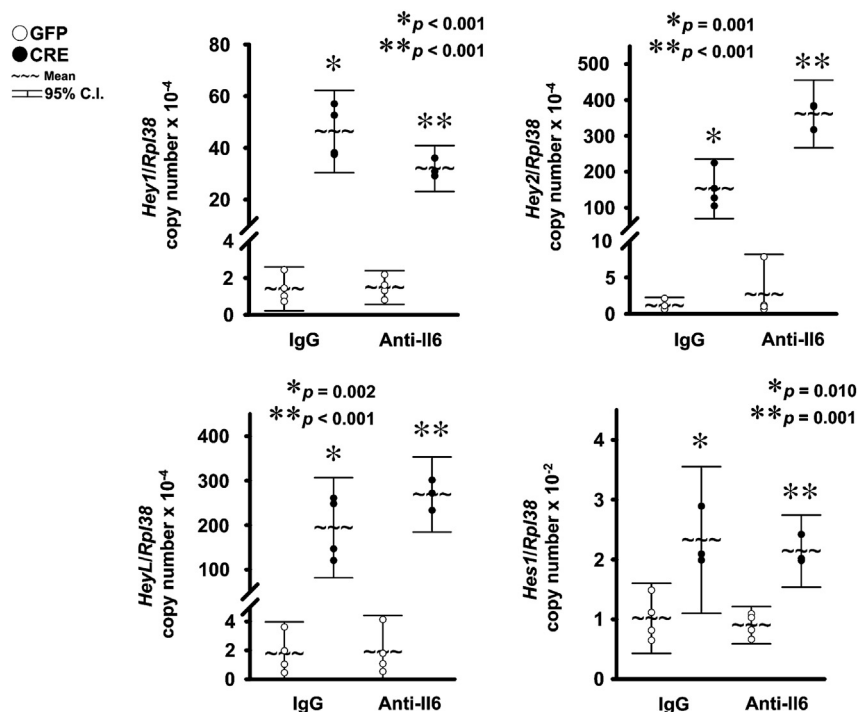


Fig. 3. Il6 neutralization does not oppose induction of Notch target genes by NICD. Primary *Rosa^{Notch}* chondrocyte-enriched cells were infected with Ad–CMV–Cre (CRE, full circles) to induce NICD or with control Ad–CMV–GFP (GFP, empty circles). Cells were cultured for 3 days under conditions favoring chondrocyte maturation and serum removed for 6 h, before exposure to murine Il6 antibody (Anti-Il6) or control normal murine IgG. Total RNA was extracted 24 h after exposure to antibodies, and changes in gene expression analyzed by qRT-PCR. Data are expressed as ratio of *Hey1*, *Hey2*, *HeyL* and *Hes1* copy number over *Rpl38* copy number. Observations represent cultures from individual mice $n = 3–4$. * indicates differences between CRE and GFP in the presence of IgG. ** indicates differences between CRE and GFP in the presence of Anti-Il6.

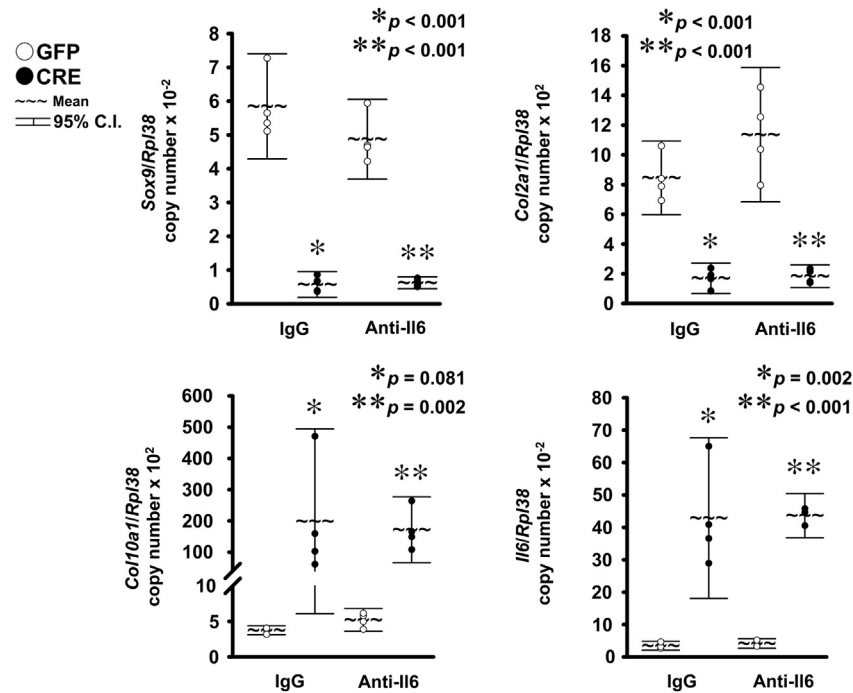


Fig. 4. Il6 neutralization does not affect *Sox9* and *Col2a1* suppression and *Col10a1* and *Il6* induction by NICD. Primary *Rosa^{Notch}* chondrocyte-enriched cells were infected with Ad–CMV–Cre (CRE, full circles) to induce NICD or with control Ad–CMV–GFP (GFP, empty circles). Cells were cultured for 3 days under conditions favoring chondrocyte maturation and serum removed for 6 h, before exposure to murine Il6 antibody (Anti-Il6) or control normal murine IgG. Total RNA was extracted 24 h after exposure to antibodies, and changes in gene expression analyzed by qRT-PCR. Data are expressed as ratio of *Sox9*, *Col2a1*, *Col10a1* and *Il6* copy number over *Rpl38* copy number. Observations represent cultures from individual mice $n = 3–4$. * indicates differences between CRE and GFP in the presence of IgG. ** indicates differences between CRE and GFP in the presence of Anti-Il6.

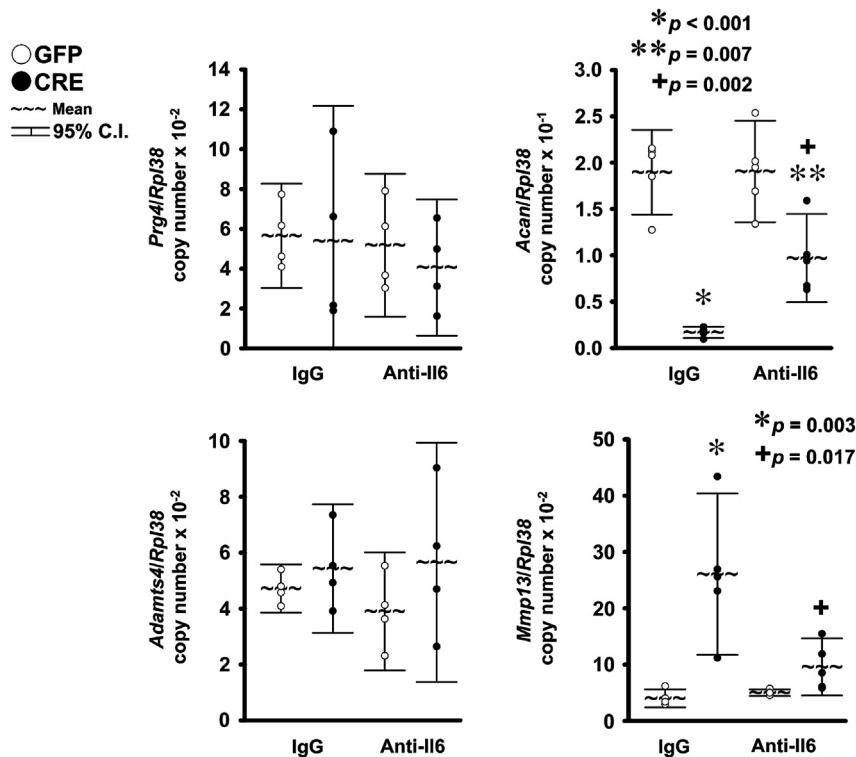


Fig. 5. Il6 neutralization opposes the effects of NICD on *Acan* and *Mmp13* expression. Primary *Rosa^{Notch}* chondrocyte-enriched cells were infected with Ad–CMV–Cre (CRE, full circles) to induce NICD or with control Ad–CMV–GFP (GFP, empty circles). Cells were cultured for 3 days under conditions favoring chondrocyte maturation and serum removed for 6 h, before exposure to murine Il6 antibody (Anti-Il6) or control normal murine IgG. Total RNA was extracted 24 h after exposure to antibodies, and changes in gene expression analyzed by qRT-PCR. Data are expressed as ratio of *Prx4*, *Acan*, *Adamts4* and *Mmp13* copy number over *Rpl38* copy number. Observations represent cultures from individual mice $n = 3–5$. * indicates differences between CRE and GFP in the presence of IgG. ** indicates differences between CRE and GFP in the presence of Anti-Il6. + indicates a difference between IgG and Anti-Il6 in the context of CRE infection.

raising the possibility that increased *Mmp13* transcripts levels exhibited by chondrocyte-enriched cell cultures overexpressing NICD is due to the presence of contaminating osteoblasts⁴⁶. However, Notch activation did not increase *Mmp13* expression in primary osteoblast cultures (data not shown), indicating that the effect is selective to cells of the chondrocyte lineage. NICD overexpression led to the induction of *Il6* and suppression of *Acan* mRNA levels, but did not affect *Prg4* or *Adamts4* expression. These findings indicate that Notch activation in murine chondrocytes affects selected gene markers of chondrocyte function and cartilage matrix degradation. It is important to mention that the model culture used contains both articular and growth plate chondrocytes, limiting our ability to discern the cell population that is responding to the Notch signal.

Notch transactivated the *Il6* promoter and induced the release of *Il6* without affecting the stability of the *Il6* mRNA, demonstrating that Notch induces *Il6* expression by transcriptional mechanisms. Activity of the *Il6* promoter and expression of *Il6* were observed in control cultures, and this is consistent with work from other laboratories reporting expression of *Il6* by chondrocytes under basal conditions⁴⁷. However, concentration of *Il6* in the supernatant of control cells was minimal and the neutralizing *Il6* antibody had no impact on chondrocyte gene markers expression in the absence of Notch, suggesting that basal levels of *Il6* are dispensable for chondrocyte function. Induction of *Il6* by Notch is in agreement with previous studies carried out in bone marrow stromal cells, macrophages and breast cancer cells, and confirms that *Il6* is a direct target gene of Notch signaling^{26,48,49}.

Activation of the Notch signaling pathway was not altered by *Il6* neutralization, excluding the possibility that the effects of *Il6* inhibition in the context of Notch induction are secondary to suppression of Notch signaling. Although *Il6* inhibited *Sox9* and *Col2a1* expression, *Il6* neutralization did not prevent suppression of both genes by Notch in *Rosa^{Notch}* chondrocyte-enriched cells, and this is in agreement with previous studies demonstrating that *HEY1* and *HES1* mediate the inhibitory effects of NICD on *COL2A1* expression in human chondrocytes^{27–29,45}. The effects of *Il6* on *Col10a1* expression were not reported, and induction of *Col10a1* by Notch was not affected by *Il6* neutralization, raising the possibility that *Col10a1*, similarly to *Hey* genes and *Hes1*, is a direct target of Notch signaling. In agreement with the effects of *Il6* on *Mmp13* in chondrocytes, *Il6* neutralization precluded *Mmp13* induction by Notch, indicating that *Il6* mediates this effect of Notch. Conversely, *Il6* neutralization partially opposed suppression of *Acan* transcripts by Notch in chondrocytes^{27–29}. *HEY1* overexpression inhibits *ACAN* expression in human bone marrow-derived chondrocytes, suggesting that suppression of *Acan* mRNA levels by Notch in murine cells is mediated by induction of both *Hey1* and *Il6*⁴⁵.

In conclusion, *Il6* mediates induction of *Mmp13* expression by Notch and contributes to the inhibitory effect of Notch on *Acan* mRNA levels in cells of the chondrocyte lineage.

Author contributions

EC obtained funding, provided substantial contributions to the design of the study, revised the manuscript critically for important intellectual content and approved the final version to be submitted. SZ obtained funding, designed the study, acquired and analyzed data, drafted and revised the article and approved the final version to be submitted. SZ takes responsibility for the integrity of the work of the work as a whole, from inception to finished article.

Role of the funding source

This work was supported by Grant DK045227 from the National Institute of Diabetes and Digestive and Kidney Diseases and Grant

AR063049 from the National Institute of Arthritis and Musculoskeletal and Skin Diseases (EC), and Research Fellowship 5371 from the Arthritis Foundation (SZ). Sponsors of this study had no role in the study design, collection, analysis and interpretation of data; in the writing of the manuscript; and in the decision to submit the manuscript for publication.

Competing interests statement

We have nothing to disclose.

Acknowledgments

The authors thank Drs D.A. Melton for *Rosa^{Notch}* mice, D.L. Allen for *Il6* promoter fragment, T. Iso for *Hey1* and *Hey2* cDNA, J.S. Nye for NICD cDNA, D. Srivastava for *HeyL* cDNA and L.J. Strobl for 12 × CSL–Luc. The authors also thank D. Bridgewater for technical assistance and M. Yurczak for secretarial assistance.

References

- Zanotti S, Canalis E. Notch and the skeleton. *Mol Cell Biol* 2010;30:886–96.
- Kopan R, Ilagan MX. The canonical Notch signaling pathway: unfolding the activation mechanism. *Cell* 2009;137:216–33.
- Kovall RA. More complicated than it looks: assembly of Notch pathway transcription complexes. *Oncogene* 2008;27:5099–109.
- Iso T, Sartorelli V, Poizat C, Iezzi S, Wu HY, Chung G, et al. HERP, a novel heterodimer partner of HES/E(spl) in Notch signaling. *Mol Cell Biol* 2001;21:6080–9.
- Olsen BR, Reginato AM, Wang W. Bone development. *Annu Rev Cell Dev Biol* 2000;16:191–220.
- Marcelino J, Carpten JD, Suwairi WM, Gutierrez OM, Schwartz S, Robbins C, et al. CACP, encoding a secreted proteoglycan, is mutated in camptodactyly-arthropathy-coxa vara-pericarditis syndrome. *Nat Genet* 1999;23:319–22.
- Pacifici M, Koyama E, Iwamoto M. Mechanisms of synovial joint and articular cartilage formation: recent advances, but many lingering mysteries. *Birth Defects Res C Embryo Today* 2005;75:237–48.
- Elsaid KA, Fleming BC, Oksendahl HL, Machan JT, Fadale PD, Hulstyn MJ, et al. Decreased lubricin concentrations and markers of joint inflammation in the synovial fluid of patients with anterior cruciate ligament injury. *Arthritis Rheum* 2008;58:1707–15.
- Ballard BL, Antonacci JM, Temple-Wong MM, Hui AY, Schumacher BL, Bugbee WD, et al. Effect of tibial plateau fracture on lubrication function and composition of synovial fluid. *J Bone Joint Surg Am* 2012;94:e64.
- Goldring MB, Marcu KB. Cartilage homeostasis in health and rheumatic diseases. *Arthritis Res Ther* 2009;11:224.
- van der Kraan PM, van den Berg WB. Chondrocyte hypertrophy and osteoarthritis: role in initiation and progression of cartilage degeneration? *Osteoarthritis Cartil* 2012;20:223–32.
- Fosang AJ, Rogerson FM. Identifying the human aggrecanase. *Osteoarthritis Cartil* 2010;18:1109–16.
- Roach HI, Yamada N, Cheung KS, Tilley S, Clarke NM, Oreffo RO, et al. Association between the abnormal expression of matrix-degrading enzymes by human osteoarthritic chondrocytes and demethylation of specific CpG sites in the promoter regions. *Arthritis Rheum* 2005;52:3110–24.
- Kapoor M, Martel-Pelletier J, Lajeunesse D, Pelletier JP, Fahmi H. Role of proinflammatory cytokines in the pathophysiology of osteoarthritis. *Nat Rev Rheumatol* 2011;7:33–42.

15. Watanabe N, Tezuka Y, Matsuno K, Miyatani S, Morimura N, Yasuda M, *et al.* Suppression of differentiation and proliferation of early chondrogenic cells by Notch. *J Bone Miner Metab* 2003;21:344–52.
16. Fujimaki R, Toyama Y, Hozumi N, Tezuka K. Involvement of Notch signaling in initiation of prechondrogenic condensation and nodule formation in limb bud micromass cultures. *J Bone Miner Metab* 2006;24:191–8.
17. Hilton MJ, Tu X, Wu X, Bai S, Zhao H, Kobayashi T, *et al.* Notch signaling maintains bone marrow mesenchymal progenitors by suppressing osteoblast differentiation. *Nat Med* 2008;14:306–14.
18. Mead TJ, Yutzev KE. Notch pathway regulation of chondrocyte differentiation and proliferation during appendicular and axial skeleton development. *Proc Natl Acad Sci USA* 2009;106:14420–5.
19. Dong Y, Jesse AM, Kohn A, Gunnell LM, Honjo T, Zuscik MJ, *et al.* RBPjkappa-dependent Notch signaling regulates mesenchymal progenitor cell proliferation and differentiation during skeletal development. *Development* 2010;137:1461–71.
20. Kohn A, Dong Y, Mirando AJ, Jesse AM, Honjo T, Zuscik MJ, *et al.* Cartilage-specific RBPjkappa-dependent and -independent Notch signals regulate cartilage and bone development. *Development* 2012;139:1198–212.
21. Zanotti S, Canalis E. Notch suppresses nuclear factor of activated T cells (NFAT) transactivation and Nfatc1 expression in chondrocytes. *Endocrinology* 2013;154:762–72.
22. Chen S, Tao J, Bae Y, Jiang MM, Bertin T, Chen Y, *et al.* Notch gain of function inhibits chondrocyte differentiation via Rbpj-dependent suppression of Sox9. *J Bone Miner Res* 2013;28:649–59.
23. Karlsson C, Brantsing C, Egell S, Lindahl A. Notch1, Jagged1, and HES5 are abundantly expressed in osteoarthritis. *Cells Tissues Organs* 2008;188:287–98.
24. Grogan SP, Miyaki S, Asahara H, D'Lima DD, Lotz MK. Mesenchymal progenitor cell markers in human articular cartilage: normal distribution and changes in osteoarthritis. *Arthritis Res Ther* 2009;11:R85.
25. Hosaka Y, Saito T, Sugita S, Hikata T, Kobayashi H, Fukai A, *et al.* Notch signaling in chondrocytes modulates endochondral ossification and osteoarthritis development. *Proc Natl Acad Sci USA* 2013;110:1875–80.
26. Sethi N, Dai X, Winter CG, Kang Y. Tumor-derived JAGGED1 promotes osteolytic bone metastasis of breast cancer by engaging notch signaling in bone cells. *Cancer Cell* 2011;19:192–205.
27. Legendre F, Dudhia J, Pujol JP, Bogdanowicz P. JAK/STAT but not ERK1/ERK2 pathway mediates interleukin (IL)-6/soluble IL-6R down-regulation of type II collagen, aggrecan core, and link protein transcription in articular chondrocytes. Association with a down-regulation of SOX9 expression. *J Biol Chem* 2003;278:2903–12.
28. Poree B, Kypriotou M, Chadjichristos C, Beauchef G, Renard E, Legendre F, *et al.* Interleukin-6 (IL-6) and/or soluble IL-6 receptor down-regulation of human type II collagen gene expression in articular chondrocytes requires a decrease of Sp1/Sp3 ratio and of the binding activity of both factors to the COL2A1 promoter. *J Biol Chem* 2008;283:4850–65.
29. Novakofski KD, Torre CJ, Fortier LA. Interleukin-1alpha, -6, and -8 decrease Cdc42 activity resulting in loss of articular chondrocyte phenotype. *J Orthop Res* 2012;30:246–51.
30. Murtaugh LC, Stanger BZ, Kwan KM, Melton DA. Notch signaling controls multiple steps of pancreatic differentiation. *Proc Natl Acad Sci USA* 2003;100:14920–5.
31. Pfander D, Cramer T, Schipani E, Johnson RS. HIF-1alpha controls extracellular matrix synthesis by epiphyseal chondrocytes. *J Cell Sci* 2003;116:1819–26.
32. Lefebvre V, Garofalo S, Zhou G, Metsaranta M, Vuurio E, de Crombrughe B. Characterization of primary cultures of chondrocytes from type II collagen/beta-galactosidase transgenic mice. *Matrix Biol* 1994;14:329–35.
33. Zanotti S, Smerdel-Ramoya A, Stadmeier L, Durant D, Radtke F, Canalis E. Notch inhibits osteoblast differentiation and causes osteopenia. *Endocrinology* 2008;149:3890–9.
34. Zanotti S, Smerdel-Ramoya A, Canalis E. Reciprocal regulation of notch and nuclear factor of activated T-cells (NFAT)c1 transactivation in osteoblasts. *J Biol Chem* 2011;286:4576–88.
35. Zanotti S, Smerdel-Ramoya A, Canalis E. Nuclear factor of activated T-cells (NFAT)C2 inhibits Notch receptor signaling in osteoblasts. *J Biol Chem* 2013;288:624–32.
36. Canalis E, Parker K, Feng JQ, Zanotti S. Osteoblast lineage-specific effects of notch activation in the skeleton. *Endocrinology* 2013;154:623–34.
37. Allen DL, Uyenishi JJ, Cleary AS, Mehan RS, Lindsay SF, Reed JM. Calcineurin activates interleukin-6 transcription in mouse skeletal muscle in vivo and in C2C12 myotubes in vitro. *Am J Physiol Regul Integr Comp Physiol* 2010;298:R198–210.
38. Nye JS, Kopan R, Axel R. An activated Notch suppresses neurogenesis and myogenesis but not gliogenesis in mammalian cells. *Development* 1994;120:2421–30.
39. Strobl LJ, Hofelmayr H, Stein C, Marschall G, Brielmeier M, Laux G, *et al.* Both Epstein–Barr viral nuclear antigen 2 (EBNA2) and activated Notch1 transactivate genes by interacting with the cellular protein RBP-J kappa. *Immunobiology* 1997;198:299–306.
40. Tso JY, Sun XH, Kao TH, Reece KS, Wu R. Isolation and characterization of rat and human glyceraldehyde-3-phosphate dehydrogenase cDNAs: genomic complexity and molecular evolution of the gene. *Nucleic Acids Res* 1985;13:2485–502.
41. Nakagawa O, Nakagawa M, Richardson JA, Olson EN, Srivastava D. HRT1, HRT2, and HRT3: a new subclass of bHLH transcription factors marking specific cardiac, somitic, and pharyngeal arch segments. *Dev Biol* 1999;216:72–84.
42. Kouadjo KE, Nishida Y, Cadrin-Girard JF, Yoshioka M, St-Amand J. Housekeeping and tissue-specific genes in mouse tissues. *BMC Genomics* 2007;8:127.
43. Bland JM, Altman DG. Multiple significance tests: the Bonferroni method. *BMJ* 1995;310:170.
44. Sokal RR, Rohlf FJ. *Biometry*. 2nd edn. 1981.
45. Grogan SP, Olee T, Hiraoka K, Lotz MK. Repression of chondrogenesis through binding of notch signaling proteins HES-1 and HEY-1 to N-box domains in the COL2A1 enhancer site. *Arthritis Rheum* 2008;58:2754–63.
46. Canalis E, Delany AM. Mechanisms of glucocorticoid action in bone. *Ann N Y Acad Sci* 2002;966:73–81.
47. Guerne PA, Carson DA, Lotz M. IL-6 production by human articular chondrocytes. Modulation of its synthesis by cytokines, growth factors, and hormones in vitro. *J Immunol* 1990;144:499–505.
48. Wongchana W, Palaga T. Direct regulation of interleukin-6 expression by Notch signaling in macrophages. *Cell Mol Immunol* 2012;9:155–62.
49. Jin S, Mutvei AP, Chivukula IV, Andersson ER, Ramskold D, Sandberg R, *et al.* Non-canonical Notch signaling activates IL-6/JAK/STAT signaling in breast tumor cells and is controlled by p53 and IKKalpha/IKKbeta. *Oncogene* 2012, <http://dx.doi.org/10.1038/onc.2012.517>.